Phosphatidic Acid Biosynthesis in Rat Liver Mitochondria and Microsomal Fractions

REGULATION OF FATTY ACID POSITIONAL SPECIFICITY

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(Received 25 February 1976)

The positional and fatty acid specificity of phosphatidic acid biosynthesis in rat liver mitochondria and microsomal fractions was studied by using acylcarnitines, CoA and an excess of carnitine palmitoyltransferase (EC 2.3.1.21) as the source of acyl-CoA. In the mitochondria, the preference for palmitic acid at the 1-position is increased at high acyl-CoA concentrations, whereas it is decreased in the microsomal fraction. There was no change in the fatty acid specificity at the 2-position with different acyl-CoA concentrations in any of the fractions. The preference in mitochondria for linoleic acid at the 2-position is strongly increased at high concentrations of lysophosphatidic acid.

The acylation of *sn*-glycerol 3-phosphate to phosphatidic acid is the initial step for the synthesis of glycerolipids *de novo* (Kornberg & Pricer, 1953; Kennedy, 1957). The acylation of glycerol phosphate takes place to about the same extent in the mitochondria and the microsomal fractions (Daae & Bremer, 1970; Sarzala *et al.*, 1970; Sánchez *et al.*, 1973; Liu & Kako, 1974; Lloyd-Davies & Brindley, 1975). The main acylation product in the mitochondria is lysophosphatidate, whereas phosphatidate is the main microsomal product (Daae & Bremer, 1970; Daae, 1972). Both the microsomal and the mitochondrial acylation of glycerol phosphate proceeds in a non-random manner. The microsomal fraction has a high preference for unsaturated fatty acids at the 2-position, whereas there is only a slight preference for saturated fatty acids at the 1-position (Okuyama & Lands, 1972). The mitochondria have a very high preference for palmitic acid at the 1-position and for unsaturated fatty acids at the 2-position (Daae, 1972; Monroy *et al.*, 1972).

Only a few studies have been performed on extrahepatic tissues. In rat heart mitochondria, Liu *et al.* (1974) found the preference for saturated and unsaturated fatty acids at the 1- and 2-positions respectively to be less pronounced than in liver mitochondria, and the positional specificity in the rat lung microsomal fraction is different from that in the liver microsomal fraction (Hendry & Possmayer, 1974). The incorporation of fatty acids into rat brain phosphatidate *in vivo* shows a different positional specificity from that in rat liver (Baker & Thompson, 1972). Whether these differences reflect the presence of organ-specific acyltransferases or merely that the observed speci-

city is modulated by factors such as the concentration of substrates or the state of the membrane into which the enzyme is embedded is not yet known. It has, however, been shown that the microsomal transferases are dependent on the presence of phospholipids (Abou-Issa & Cleland, 1969).

In the rat liver microsomal fraction the observed specificity of the acyl-CoA-1-acylglycerol phosphate acyltransferase is lost at high concentrations of 1-acylglycerol phosphate (Okuyama & Lands, 1972), indicating that substrate concentrations might regulate the fatty acid specificity of glycerol phosphate acylation. The effect of substrate concentrations on the mitochondrial acyltransferase(s) has not, however, been reported. We have therefore compared the acylation of glycerol phosphate in rat liver mitochondria and microsomal fractions in this respect.

Experimental

General

Male Wistar rats (180–220 g) fed *ad libitum* were used. Rat liver mitochondria and microsomal fractions were prepared as described previously (Daae & Bremer, 1970). With this procedure the mitochondrial preparations contained 7–18% of microsomal protein, calculated from the distribution of glucose 6-phosphatase (EC 3.1.3.9). *rac*-Glycerol 3-phosphate (sodium salt), palmitic acid and linoleic acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; CoA was from C. F. Boehringer and Sons, Mannheim, Germany; [9,10-³H]palmitic acid and [1-¹⁴C]linoleic acid were from The Radiochemical Centre, Amersham, Bucks., U.K. A partly

purified phospholipase A from *Vipera russelli* venom was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. A pure, crystalline phospholipase A from pig pancreas was the kind gift of Professor de Haas, State University of Utrecht, The Netherlands. [9,10-³H]Palmitoylcarnitine and [1-¹⁴C]linoleoylcarnitine were prepared as described by Christophersen & Bremer (1972). The specific radioactivity of [9,10-³H]palmitoylcarnitine was 24.6 $\mu\text{Ci}/\mu\text{mol}$ and that of [1-¹⁴C]linoleoylcarnitine 2.95 $\mu\text{Ci}/\mu\text{mol}$. Carnitine palmitoyltransferase (EC 2.3.1.21) was prepared as described by Farstad *et al.* (1967).

Incubations

The basic incubation mixture contained 130 nmol of [9,10-³H]palmitoylcarnitine; 130 nmol of [1-¹⁴C]linoleoylcarnitine, 10 μmol of GSH; 30 μmol of Tes[N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid] buffer pH 7.4, 20 μmol of KF, carnitine palmitoyltransferase corresponding to 3.2 mg of protein, and 0.15 M-KCl, in a total volume of 2.0 ml. CoA, *rac*-glycerol 3-phosphate, mitochondria and microsomal fractions were added as indicated in the legends to the Tables. Albumin was omitted, to avoid excessive accumulation of lysophosphatidic acid (Monroy *et al.*, 1972). The addition of KF decreased the formation of monoacylglycerols and diacylglycerols and increased the production of phosphatidic acid, probably owing to inhibition of the phosphatidate phosphohydrolase (EC 3.1.3.4). The incubations were started by the addition of either mitochondria or microsomal fractions, and performed in a shaking water bath at 30°C for 3 min.

Isolation of phosphatidic acid

Reactions were stopped by adding 3.0 ml of butanol-1-ol followed by 4 ml of 0.1 M-Tes buffer (pH 7.4). The butanol phase was removed, and the water phase re-extracted with 4 ml of butanol. The combined butanol phases were washed with 4 ml of Tes buffer and then 1 mg of butylated hydroxytoluene was added as an antioxidant. The lipids were evaporated to dryness under N₂ and then chromatographed on plates prepared from 62.6 mg of EDTA (dipotassium salt), 64 g of silica gel H (E. Merck, Darmstadt, W. Germany) and 155 ml of water and then activated for 1 h at 120°C. The solvent was chloroform/methanol/acetic acid/water (73:20:4:3, by vol.) containing 50 mg of butylated hydroxytoluene/litre. The phosphatidic acid was localized by counting for radioactivity after scraping a narrow band in 0.5 cm-wide zones into counting vials. The zone corresponding to phosphatidic acid was transferred to sintered glass funnels, and the gel eluted as described by Arvidson (1968).

Fatty acid positional analysis

The phosphatidic acid was hydrolysed with a partly purified phospholipase A₂ from *Vipera russelli* venom. The lipid was dissolved in 10 ml of diethyl ether and then 5 mg of phosphatidylcholine prepared from egg yolk as described by Dawson (1963) was added. Next, 1 ml of calcium borate buffer (0.1 M-boric acid and 5 mM-CaCl₂), pH 7.2, and phospholipase corresponding to 0.2 mg of protein were added and the mixture was incubated at room temperature (20°C) for 4 h in glass-stoppered tubes with continuous shaking. The ether phase was pipetted off, and the water phase extracted with 3 ml of butanol-1-ol and then 4 ml of 0.01 M-HCl was added. The butanol phase was removed and the water phase re-extracted with 3 ml of butanol. The combined butanol phases were washed once with 3 ml of 0.1 M-HCl saturated with butanol, and then combined with the ether extract. The hydrolysis products were separated and localized as described above, and the lipids eluted with 3 $\times$ 4 ml portions of chloroform/methanol/acetic acid/water (50:39:1:10, by vol.) directly into counting vials. The eluates were evaporated to dryness and dissolved in 0.5 ml of methanol before radioactivity counting. The percentage of palmitic acid and linoleic acid at each position was calculated from the specific radioactivities of the acylcarnitines and the amount of label present at each position.

Precision of fatty acid positional analysis

In some experiments, portions of phosphatidic acid were also hydrolysed with a pure, crystalline lipase as described by de Haas *et al.* (1968). Identical results were obtained with the two methods, the degree of hydrolysis being slightly less with *Vipera russelli* venom than with the pancreatic lipase, but being in the range of 75–95% for both. With the *Vipera russelli* venom, 14 separate analyses were performed on the same batch of radioactively labelled phosphatidic acid. In the 1-position was found 74.0% palmitic acid and 26.0% linoleic acid, the coefficients of variation being 3.7 and 10.5% respectively. At the 2-position was found 16.3% palmitic acid and 83.7% linoleic acid, the coefficients of variation being 15.4 and 3.0% respectively. There was no change in the observed positional specificity at either position, with different degrees of hydrolysis ranging from 34–84%. No corrections were made for any non-enzymic hydrolysis, which would tend to decrease any positional specificity.

General analytical procedures

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Radioactivity was determined with a Packard Tri-Carb 3385 liquid-scintillation spectrometer, by using a dioxan-based scintillation fluid (Bjerve & Bremer,

1969). The counting efficiency was determined by using external standard ratio. For all counting efficiencies the spill-over of ^3H into the ^{14}C channel was less than 0.06%. The spill-over of ^{14}C into the ^3H channel varied between 14 and 30%, depending on the degree of quenching.

Results and Discussion

Effect of CoA on fatty acid specificity

In the experiments presented here, equimolar concentrations of palmitoylcarnitine and linoleoylcarnitine in the presence of CoA and acyl-CoA-carnitine acyltransferase were used as the source of acyl-CoA. Increasing the CoA concentration in this

system will consequently give an increased concentration of acyl-CoA available for glycerol phosphate acylation. It has been shown that the carnitine acyltransferase is nearly equally active towards palmitoyl- and linoleoyl-carnitine (Christophersen & Bremer, 1972). Further, a very high excess of the carnitine palmitoyltransferase was used, which would tend to eliminate any small differences in reactivity towards the two acyl-CoA esters.

Tables 1 and 2 show the effect of increasing CoA concentrations on the fatty acid specificity in rat liver mitochondria and microsomal fractions. The rate of phosphatidic acid synthesis corresponds well to that reported with unlabelled acylcarnitines (Daae & Bremer, 1970). Linoleic acid is incorporated 10–20

Table 1. *Effect of CoA on fatty acid specificity in mitochondrial glycerol phosphate acylation*

The basic incubation medium contained 10mM-*rac*-glycerol 3-phosphate and CoA as indicated. The reaction was started by adding 7.8 mg (Expt. I), 8.0 mg (Expt. II) or 7.4 mg (Expt. III) of mitochondrial protein. The amount of [9,10- ^3H]palmitic acid ($\text{C}_{16:0}$) and [1- ^{14}C]linoleic acid ($\text{C}_{18:2}$) incorporated into each position is given in nmol, with the percentage distribution of the two fatty acids at each position given in parentheses. The values in Expt. III are from duplicate experiments.

Expt. no. ...	Position 1								Position 2							
	I		II		III				I		II		III			
	$\text{C}_{16:0}$	$\text{C}_{18:2}$	$\text{C}_{16:0}$	$\text{C}_{18:2}$	$\text{C}_{16:0}$	$\text{C}_{18:2}$	$\text{C}_{16:0}$	$\text{C}_{18:2}$	$\text{C}_{16:0}$	$\text{C}_{18:2}$	$\text{C}_{16:0}$	$\text{C}_{18:2}$	$\text{C}_{16:0}$	$\text{C}_{18:2}$	$\text{C}_{16:0}$	$\text{C}_{18:2}$
12.5	2.3 (67)	1.1 (33)	3.0 (59)	2.1 (41)	1.1 (72)	0.4 (28)	1.3 (77)	0.4 (23)	0.3 (9)	3.1 (91)	0.2 (4)	4.8 (96)	0.4 (29)	1.1 (71)	0.1 (8)	1.6 (92)
125	5.0 (83)	1.1 (17)	8.4 (88)	1.2 (12)	4.1 (86)	0.7 (14)	3.4 (85)	0.6 (15)	0.6 (9)	5.5 (91)	1.3 (14)	8.3 (86)	0.5 (10)	4.3 (90)	0.4 (11)	3.5 (89)
500	3.9 (94)*	0.2 (6)	6.8 (88)*	1.0 (12)	5.4 (91)*	0.5 (9)	5.5 (89)*	0.7 (11)	0.5 (13)	3.6 (87)	0.5 (7)	7.3 (93)	0.5 (9)	5.4 (91)	1.1 (18)	5.1 (82)

* A Kruskal-Wallis rank test (Lehmann, 1975) showed that the results are different from those at the lowest CoA concentration ($P < 0.01$).

Table 2. *Effect of CoA on fatty acid specificity in microsomal glycerol phosphate acylation*

The basic incubation medium contained 10mM-*rac*-glycerol 3-phosphate and CoA as indicated below. The reaction was started by adding 4.6 mg (Expt. I) or 4.0 mg (Expt. II) of microsomal protein. For details see Table 1. Values in Expt. II are from duplicate experiments.

Expt. no. ...	Position 1						Position 2					
	I		II				I		II			
	$\text{C}_{16:0}$	$\text{C}_{18:2}$	$\text{C}_{16:0}$	$\text{C}_{18:2}$	$\text{C}_{16:0}$	$\text{C}_{18:2}$	$\text{C}_{16:0}$	$\text{C}_{18:2}$	$\text{C}_{16:0}$	$\text{C}_{18:2}$	$\text{C}_{16:0}$	$\text{C}_{18:2}$
12.5	3.8 (64)	2.2 (36)	7.1 (83)	1.5 (17)	6.3 (81)	1.5 (19)	0.3 (6)	5.7 (94)	1.7 (20)	6.9 (80)	1.1 (14)	6.7 (86)
125	6.4 (47)	7.1 (53)	—	—	—	—	1.1 (8)	12.3 (92)	—	—	—	—
500	7.2 (43)*	9.6 (57)	20.0 (69)*	9.2 (31)	21.2 (75)*	7.1 (25)	0.5 (3)	16.2 (97)	3.1 (11)	26.0 (89)	5.6 (20)	22.7 (80)

* A *t* test was performed to test the hypothesis that there is no change in fatty acid specificity at position 1 at CoA concentrations above or below 100 μM . The variance was estimated from 14 separate analyses (see the Experimental section). The hypothesis was rejected at the level of $P < 0.05$.

times faster than palmitic acid into the 2-position in both fractions. The relative incorporation rates of these two fatty acids is not dependent on the concentration of CoA. This confirms that the ratio of palmitoyl-CoA to linoleoyl-CoA formed by the carnitine palmitoyltransferase is not dependent on the concentration of CoA.

At the 1-position, however, the fatty acid specificity is clearly dependent on the CoA concentration. Most probably, this is not an effect of CoA itself, but reflects the changes in acyl-CoA concentration. At the lowest CoA concentrations, palmitic acid is incorporated 2–4 times faster than linoleic acid into the 1-position in both subcellular fractions. On increasing the CoA concentration in the mitochondria, the preference for palmitic acid is highly increased. Thus, at 0.5 mM-CoA, palmitic acid is incorporated 10–20 times faster than linoleic acid. A Kruskal-Wallis rank test (Lehmann, 1975) showed that the specificity for palmitic acid at the 1-position at 500 μ M-CoA was significantly ($P < 0.01$) higher than at 12.5 μ M. In the microsomal preparations, the incorporation of linoleic acid was increased more than that of palmitic acid, resulting in a low degree of specificity at the 1-position at the highest CoA concentrations.

It has been reported that in the mitochondria the first fatty acid is introduced at the 1-position, and that palmitic acid is incorporated much faster than oleic acid and linoleic acid into this position, whereas linoleic acid is preferentially incorporated into the 2-position (Daae, 1972; Monroy *et al.*, 1972). In the microsomal fraction it has clearly been shown that

the first acylation takes place at the 1-position (Okuyama *et al.*, 1971; Tamai & Lands, 1974). There is a very high preference for unsaturated fatty acids at the 2-position, whereas it has been disputed whether there is any preference for saturated fatty acids at the 1-position in the microsomal fraction (Possmayer *et al.*, 1969; Okuyama & Lands, 1972). The results reported here correspond well to those mentioned above, and also show that the fatty acid specificity of the enzyme(s) introducing the first fatty acid depends on the concentration of acyl-CoA. This can explain some of the discrepancies in previous publications on positional and fatty acid specificity in glycerol phosphate acylation.

In the present study, the specificity for palmitic acid at the 1-position is increased in the mitochondria and decreased in the microsomal fraction by increasing the concentration of acyl-CoA. This is puzzling, in view of the results of Yamashita & Numa (1972), who isolated a glycerol phosphate acyltransferase from the rat liver microsomal fraction. This transferase had a high preference for palmitic acid, which became more pronounced at high acyl-CoA concentrations. Whether this discrepancy is due to the presence of more than one acyltransferase acting at the 1-position in the microsomal fraction, or whether the observed transferase specificity is totally dependent on intact membrane structures (Abou-Issa & Cleland, 1969), is not yet known.

Effect of glycerol phosphate on fatty acid specificity

Tables 3 and 4 show the effect of varying the con-

Table 3. *Effect of glycerol phosphate on fatty acid specificity in mitochondrial glycerol phosphate acylation*

The basic incubation medium contained 0.125 mM-CoA and *rac*-glycerol 3-phosphate as indicated below. The reaction was started by adding 7.8 mg (Expt. I), 7.4 mg (Expt. II) or 8.0 mg (Expt. III) of mitochondrial protein. For details see Table 1.

Expt. no. ...	Position 1						Position 2					
	I		II		III		I		II		III	
	C _{16:0}	C _{18:2}	C _{16:0}	C _{18:2}	C _{16:0}	C _{18:2}	C _{16:0}	C _{18:2}	C _{16:0}	C _{18:2}	C _{16:0}	C _{18:2}
<i>rac</i> -Glycerol phosphate (mM)												
0.5	3.5 (92)	0.3 (8)	1.6 (97)	0.1 (3)	3.4 (98)	0.1 (2)	1.3 (34)	2.5 (66)	0.8 (48)	0.9 (52)	0.9 (27)	2.6 (73)
2.5	3.6 (79)	0.9 (21)	—	—	—	—	0.4 (8)	4.2 (92)	—	—	—	—
3.0	—	—	3.4 (100)*	0 (0)*	6.8 (90)	0.8 (10)	—	—	1.1 (32)	2.3 (68)	1.2 (16)	6.3 (84)
5.0	3.6 (73)	1.3 (27)	—	—	—	—	0.4 (8)	4.5 (92)†	—	—	—	—
10.0	—	—	6.9 (90)	0.7 (10)	9.5 (90)	1.0 (10)	—	—	1.9 (24)	5.8 (76)†	0.8 (7)	9.7 (93)†

* Estimated from very low c.p.m. values owing to loss of material.

† A *t* test was performed to test the hypothesis that there was no difference in fatty acid specificity at position-2 between 0.5 mM-glycerophosphate and 5 or 10 mM-glycerophosphate. The hypothesis was rejected at a level of $P < 0.01$.

Table 4. *Effect of glycerol phosphate on fatty acid positional specificity in microsomal glycerol phosphate acylation*

The basic incubation medium contained 0.125 mM-CoA and *rac*-glycerol 3-phosphate as indicated below. The reaction was started by adding 4.6 mg (Expt. I) or 4.0 mg (Expt. II) of microsomal protein. For details see Table 1.

<i>rac</i> -Glycerol phosphate (mM)	Expt. no. ...	Position 1				Position 2			
		I		II		I		II	
		C _{16:0}	C _{18:2}	C _{16:0}	C _{18:2}	C _{16:0}	C _{18:2}	C _{16:0}	C _{18:2}
0.5		5.0 (59)	3.5 (41)	1.9 (38)	3.1 (62)	0.5 (6)	8.0 (94)	0.2 (4)	4.8 (96)
3.0		15.9 (66)	8.0 (34)	4.7 (53)	4.2 (47)	2.6 (11)	21.3 (89)	1.2 (13)	7.8 (87)
10.0		14.7 (67)	7.3 (33)	5.7 (51)	5.6 (49)	3.3 (15)	18.8 (85)	0.5 (5)	10.8 (95)

centrations of glycerol phosphate on the fatty acid specificity in mitochondria and microsomal fractions. The CoA concentration was high enough to give a pronounced preference for palmitic acid at the 1-position in the mitochondria. The second acylation of glycerol phosphate is the rate-limiting step in phosphatidic acid biosynthesis in the mitochondria, but it is not in the microsomal fraction (Daae & Bremer, 1970). Monroy *et al.* (1972) have shown that lysophosphatidic acid disappears when mitochondria are incubated with 0.15 mM-*sn*-glycerol 3-phosphate, whereas a continuous accumulation of lysophosphatidic acid was observed at 15 mM-glycerol phosphate. In the present albumin-free system, 0.3 nmol of lysophosphatidate was formed/mg of mitochondrial protein at 0.5 mM-*rac*-glycerol 3-phosphate compared with 3.6 nmol at 10 mM-glycerol phosphate. In the presence of 0.5% albumin, the corresponding values were 3.3 and 9.5 nmol/mg of mitochondrial protein. No similar accumulation has been shown in the microsomal fraction (Daae & Bremer, 1970). As the microsomal capacity for phosphatidic acid biosynthesis in this system is higher than that of mitochondria, the amount of microsomal protein used was about half of that in the mitochondrial experiments.

Increasing glycerol phosphate concentrations did not affect the fatty acid specificity at the 1-position in mitochondria significantly. In the microsomal fraction, the incorporation of palmitic acid into the 1-position was stimulated to a greater degree than that of linoleic acid. Glycerol phosphate had no effect on the microsomal preference for linoleic acid at the 2-position. It has been reported that high concentrations of 1-lysophosphatidic acid abolish the fatty acid specificity at this position (Okuyama & Lands, 1972). However, the high capacity for the second acylation will probably prevent any significant accumulation of this intermediate, even at the highest glycerol phosphate concentrations used. In the mitochondria,

however, increasing glycerol phosphate concentrations increase the preference for linoleic acid at the 2-position significantly ($P < 0.01$). This is probably not due to glycerol phosphate itself, as the latter has no or very little effect on the other acyltransferases in this assay system. It rather indicates that the preference for linoleic acid increases with the concentration of 1-lysophosphatidic acid.

Metabolic significance

Although the mitochondrial capacity for phosphatidic acid biosynthesis has been disputed (Eibl *et al.*, 1969; Davidson & Stanacev, 1972), it seems now settled that the mitochondria possess one-third to two-thirds of the capacity of the total cell homogenate (Shephard & Hübscher, 1969; Zborowski & Wojtczak, 1969; Daae & Bremer, 1970). Thus the mitochondria will contribute significantly to the observed distribution of fatty acids in naturally occurring glycerolipids. Phosphatidic acid isolated from rat liver contains palmitic acid as the main fatty acid at the 1-position (Åkesson *et al.*, 1970). As the microsomal fraction seems to have a low fatty acid specificity at the 1-position, this could indicate that phosphatidic acid and/or lysophosphatidic acid produced in the mitochondria can be transported to other cell organelles, i.e. the microsomal fraction where they can act as precursors for other glycerolipids. Another possibility is a preferential microsomal biosynthesis of 1-palmitoylglycerol phosphate via the dihydroxyacetone phosphate pathway (Hajra, 1970). However, it is disputed whether this pathway plays any role in the biosynthesis of phosphatidic acid (Manning & Brindley, 1972; Rognstad *et al.*, 1974; Pollock *et al.*, 1975), and the biosynthesis of acyl-dihydroxyacetone phosphate takes place predominantly in the mitochondria (LaBelle & Hajra, 1972).

The present results strongly indicate that the concentrations of both acyl-CoA and lysophosphatidic acid influence the pattern of fatty acid incorporation

into phosphatidic acid, and also confirm that the mitochondrial acyltransferases are different from the microsomal ones (Daae, 1972; Monroy *et al.*, 1972; Okuyama & Lands, 1972). The results also indicate that the mitochondrial acyltransferases acting at the 1-position have different K_m values for palmitic acid and linoleic acid, and that lysophosphatidic acid can change the apparent K_m value differently for the two fatty acids at the 2-position. The fatty acid and positional specificity in glycerol phosphate acylation *in vivo* will therefore presumably be different in animals in different nutritional states (fed, starved or ketotic) as this will affect the tissue concentrations of both acyl-CoA and glycerol phosphate (Bortz & Lynen, 1963; Böhmer, 1967).

[9,10-³H]Palmitoylcarnitine and [1-¹⁴C]linoleoylcarnitine were synthesized by Dr. B. O. Christophersen. Dr. Steinar Bjerme gave helpful advice on the statistical treatment of the data. The skilled technical assistance of Mrs. Ann Olsen and Miss Live Horn is highly appreciated. This work was supported by the Norwegian Cancer Society.

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